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Most neuropeptides are incapable of entering brain from blood owing to the presence of unique anatomical structures in the brain capillary wall, which makes up the blood-brain barrier (BBB). Such neuropeptides could be introduced into the bloodstream by intranasal insufflation and, thus, could have powerful medicinal properties (e.g., β-endorphin for the treatment of pain, vasopressin analogues for treatment of memory, ACTH analogues for treatmen of post-traumatic epilepsy), should these peptides be capable of traversing the BBB. One suc strategy for peptide delivery through the BBB is the development of chimeric peptides, which is the basis of the present contract. The production of chimeric peptides involves the covalent coupling of a nontransportable peptide (e.g., β-endorphin, vasopressin) to a transportable vector peptide (e.g., insulin, transferrin, cationized albumin, histone). The transportable peptide is capable of penetrating the BBB via receptor-mediated or absorptive-mediat transcytosis. Therefore, the introduction of chimeric peptides allows the nontransportable peptide to traverse the BBB via physiologic mechanisms by "piggy back" on the transportable
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18. SUBJECT TERMS (continued from reverse side)

memory enhancement, ACTH (adrenocorticotrophic hormone), MSH (melanocyte-stimulating hormone), AIDS (autoimmune deficiency syndrome), SPDP (N-succinimidyl 3-(2-pyridyldithio)proprionate, LDL (low density lipoprotein).

19. ABSTRACT (continued from reverse side)

vector. The work of the present contract is based in four different areas: synthesis of chimeric peptides, physiologic studies to demonstrate transportability of the chimeric peptide across the rat BBB in vivo, morphologic studies using either electron microscopic organelles involved in chimeric peptide trafficking through brain capillary endothelial cytoplasm, and biochemical studies aimed at purifying the subcellular vesicles involved in neuropeptide transcytosis across the BBB. The work of the first eighteen months of this contract demonstrates that the development of chimeric peptides is highly feasible and that these products do act as vehicles for transport of neuropeptides such as β-endorphin through the BBB. Thus, this approach may prove to be efficacious in the future for delivery of medicinal substances into the human brain.

FOREWORD

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Cynthia M. Sanders M.D. 1/30/89
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INTRODUCTION

The developments in molecular neurobiology are allowing neurologic and psychiatric disorders to be defined in molecular terms (1). With the advent of this new knowledge will come the development of pharmaceutical compounds that are tailored to a specific disease on the basis of the molecular biology of that particular disorder. Invariably, such therapeutic compounds will be specific neuropeptides that are deficient in certain areas of the brain and the correction of this deficiency could allow for treatment of the neurologic or mental disorders. Possible disorders and the respective peptides that may be efficacious in the illness include: epilepsy (ACTH analogues), fever (MSH analogues), pain (β-endorphin), brain tumors (interleukins, monoclonal antibodies), depression (CRH antagonists), or cerebral AIDS (CD4 antigen) (1-7). In addition, neuropeptides may also be used to optimize brain performance: memory (e.g., vasopressin analogues) (8).

Unfortunately, none of the above peptides are capable of traversing the brain capillary wall which makes up the blood-brain barrier (BBB) in vivo. The microvessels of all organs, except for the brain, are endowed with small pores that allow for small molecules such as neuropeptides to readily escape from blood into the respective organ (9). However, in the brain of all vertebrates, these microvascular pores are completely absent and, thus, circulating neuropeptides do not gain access to brain interstitial space from the circulation (10). Since neuropeptides may be administered into the bloodstream conveniently by intranasal insufflation (11), the normal gut barriers to peptides may be easily traversed and the rate-limiting step in the development of peptides as neuropharmaceuticals is circumvention of the BBB.

There are three possible strategies for drug and neuropeptide delivery through the BBB (12): neurosurgical or invasive-based strategies, lipidization or pharmacologic-based strategies, and chimeric peptide or physiologic-based strategies. Of the neurosurgical-based strategies, there are two possibilities. First, attempts have been made to transport drugs across the BBB after intracarotid injections of hypertonic solutions (13). However, this is a highly invasive procedure that has a 15-20% side effect of seizures in experimental animals, must be performed in an operating room under heavy sedation, and is obviously not amenable to everyday use. The second type of neurosurgical or invasive strategy is the implantation of a chronic intraventricular catheter (14). However, this approach is limited by the fact that the pharmaceutical agent is only delivered to the surface of the brain with this approach. Owing to the fact that cerebrospinal fluid is pumped out of the brain faster than molecules can diffuse down into brain parenchyma, the delivery of neuropeptides into brain CSF by the catheter route would not allow for treatment of neurologic diseases that are not meningeal-based in etiology. The lipidization strategy involves converting water soluble drugs into lipid soluble pro-drugs. However, this approach cannot be used for neuropeptides. Recent studies from the Principal Investigator's laboratory have shown that cyclosporin, which is an extremely lipid soluble peptide, is not capable of crossing the BBB owing to it's molecular size of 1,100 Daltons (15).

Therefore, even if the highly hydrophillic peptides were made lipid soluble, their large molecular weight would preclude their transport through the BBB. Thus, the only apparent strategy known today for delivery of neuropeptides through the BBB is the development of chimeric peptides.

The development of chimeric peptides involves the coupling of a nontransportable peptide (e.g., β-endorphin) to a transportable peptide (e.g., insulin, transferrin, cationized albumin, or histone). The transportable peptide vector is a molecule that is known to be transported through the BBB via either receptor-mediated (e.g., insulin, transferrin) or absorptive-mediated (e.g., cationized albumin, histone) transcytosis through the endothelial cytoplasm. First generation chimeric peptides involves covalent coupling of a nontransportable and transportable peptide using disulfide-based cross-linking reagents such as SPDP. Second generation chimeric peptides will undoubtedly involve recombinant DNA technology wherein genes of the transportable and nontransportable peptides are fused and the entire recombinant chimeric peptide is produced in large quantities in either prokaryotic or eukaryotic expression systems.

The aims of the present contract are four-fold: to synthesize chimeric peptides, to demonstrate their transport into brain interstitium by physiologic-based methods, to demonstrate morphologically the subcellular endothelial organelles involved in chimeric peptide trafficking, and to purify these vesicles with biochemical techniques.

EXPERIMENTAL METHODS

I. CHIMERIC PEPTIDE SYNTHESIS

Beta-endorphin and [D-Ala^2]β-endorphin (DABE) have been covalently coupled to either cationized albumin or to histone using the disulfide-based coupling reagent SPDP (16). Primary amino groups on the transport vector are coupled to primary amino groups on β-endorphin or DABE-lysine moieties using the coupling reagent (17). The use of SPDP is advantageous since the chimeric peptide can be split by reducing agents such dithiothreitol in vitro or by glutathione-dependent enzymes in brain in vivo.

Radioreceptor Assay In Vitro

The ability of the chimeric peptide to circumvent the BBB is initially screened with a convenient in vitro assay called a radioreceptor assay. This assay uses isolated bovine brain microvessels as an in vitro assay of BBB transport. The binding and internalization of the peptides are determined with this preparation in vitro as described previously (17-19).

II. PHYSIOLOGIC-BASED METHODS

The transport of chimeric peptides into brain interstitium in vivo is demonstrated with both qualitative methods, such as autoradiography (see below), and quantitative physiologic-based methods. During the initial year of the contract, we developed an

internal carotid artery perfusion/capillary depletion method for quantifying BBB transcytosis of potential transport vectors and chimeric peptides. The exact details of the internal carotid artery perfusion/capillary depletion technique have been described previously (20). Initially, this method was used to show that two potential transport vectors such as cationized albumin or cationized immunoglobulin G, but not acetylated LDL, as transported through the BBB. The internal carotid artery/capillary depletion technique has also recently been used to document the use of histone as a potential transport vector (manuscript in preparation). The development of the internal carotid artery/capillary depletion method has been an important development in the first year of funding of this contract, since heretofore there had not been a suitable quantitative method for demonstrating peptide transcytosis into brain. It is not adequate to simply perfuse radiolabeled peptide into the carotid artery and measure the uptake by brain of the peptide as compared to non-transportable substances such as albumin. This is because we have found that many substances are simply bound and sequestered by the microvasculature without actually traversing the capillary barrier and entering brain interstitial space. A prime example of this is acetylated LDL (20) and β-endorphin (manuscript in preparation). We believe this new internal carotid artery perfusion/capillary depletion method is a powerful new tool. The University of California has filed a patent on this technique and this disclosure has previously been made to the Department of Defense.

III. MORPHOLOGIC-BASED METHODOLOGIES

A. Autoradiography

We initially proposed to perform autoradiography at both the light and electron microscopic levels to document peptide transcytosis into brain. The light microscopy autoradiography studies have been performed previously (17, 19). We subsequently initiated electron microscopic autoradiography. In these studies we perfused a [^{125}I]-transport vector into the carotid artery of an anesthetized rat for ten minutes, followed by flushing to clear out radioactive contents of the cerebrovasculature, followed by in situ perfusion fixation with glutaraldehyde for electron microscopy processing. Following embedding in Epon and thin-sectioning, the grids were coated with Kodak emulsion and developed for 4-6 weeks. Thick (1-micron) sections were also obtained for light microscopy. The light microscopy demonstrated suitable silver grains in the parenchyma and we subsequently developed electron microscopic autoradiography. However, at this point, our results, plus our reading of the literature, led to the conclusion that autoradiography at the electron microscopic level was not suitable for identifying the subcellular endothelial organelles involved in peptide transcytosis. This is because the [^{125}I]-silver grain covers a distance of ~0.1 micron, and the thickness of the entire capillary endothelial cells is only ~0.3 micron. Therefore, the silver grain would completely obliterate any subcellular organelles. On this basis, the P.I. and the Postgraduate Researcher, Dr. Catherine Farrell, have developed electron microscopic immunogold methods.

B. Electron Microscopic Immunogold Methods

The use of 15 nm gold particles to tag the chimeric peptide or transport vector within the endothelial cytoplasm is a more suitable approach than autoradiography, since the 15 nm gold particle would be smaller than the subcellular endothelial organelles involved in peptide trafficking. These methods are just now being developed but, briefly, the animal is infused with a transport vector (e.g., cationized immunoglobulin), followed by perfusion fixation with paraformaldehyde, followed by embedding in water soluble resins such as LR-white, followed by thin-sectioning. The thin sections are then applied to a primary antiserum (e.g., a rabbit antiserum directed against cationized bovine immunoglobulin, which is being prepared in our laboratory). The primary antibody is then tagged with a goat anti-rabbit antibody that is conjugated to 15 nm gold particles suitable for visualization with electron microscopy. The immunogold is often inspected at the light microscopic level with the silver enhancement technique using thick (1 micron) sections.

IV. BIOCHEMICAL ISOLATION OF TRANSPORT VESICLES

The isolation of the subcellular endothelial organelles involved in peptide transcytosis through the blood-brain barrier cannot be attempted until these vesicles are actually identified by the electron microscopic immunogold methods detailed above. However, in preparation of these studies we have initiated preliminary attempts to purify endothelial subcellular vesicles. Initially, we sonicated isolated brain capillaries and then separated the subcellular organelles on Percoll density gradients. However, measurement of endothelial enzymes indicated that the highly disruptive sonication step prevented precise separation of endothelial organelles. On this basis, we have replaced the sonication step with a multiple syringe homogenization technique and we are presently perfecting these methods.

CONCLUSIONS

The work of the first eighteen months of this contract has clearly demonstrated the feasibility of the chimeric peptide approach for solving the problem of neuropeptide delivery through the BBB (21). Thus, the present work has been an important first step in realizing the longterm goal of ultimately using these substances for medicinal purposes for the human brain. The specific accomplishments of the first eighteen months are as follows:

1. The development of an internal carotid artery perfusion/capillary depletion method which allows, for the first time, for the quantification of peptide transcytosis through the BBB in vivo (20).
2. The first synthesis of a chimeric peptide (β-endorphin coupled to cationized albumin) and the first demonstration that chimeric peptides can be successfully used to deliver nontransportable peptides through the BBB (17).

3. The first demonstration of an insulin-like growth factor (IGF) receptor on the human blood-brain barrier (18). This suggests that IGF-1, but more likely IGF-2, are potential transport vectors in future chimeric peptide synthesis.
4. The first demonstration that cationized immunoglobulin G is capable of traversing the BBB via absorptive-mediated transcytosis, whereas native IgG is incapable of crossing the barrier (18). Thus, cationized immunoglobulin G is potential transport vector that could be relatively brain-specific. For example, the use of a monoclonal antibody to the brain capillary that is cationized may be a more brain-specific transport vector than all the vectors known thus far. In addition, since cationization does not destroy immunoglobulin G antigenicity, the use of cationized immunoglobulins may be used for neuroimaging diagnostic purposes. This could not have been done previously since native immunoglobulin G molecules do not cross the BBB.
5. The first demonstration that histone is a suitable endogenous polycationic protein that can be used as a potential BBB transport vector (manuscript in preparation, to be submitted with the April 1, 1989 Progress Report). Since the other polycationic proteins such as cationized albumin or cationized immunoglobulin G may cause the formation of antibodies in human subjects, the use of endogenous polycations such as histone may be advantageous since it would not be expected to develop antibodies.

There are no substantial changes from the initial specific aims of the original contract application with the exception of replacing electron microscopic autoradiography with electron microscopic immunogold studies. The goals of the remaining eighteen months of the contract are as follows:

1. Complete present studies demonstrating transcytosis of DABE chimeric peptide, through the BBB in vivo.
2. Initiate studies involving synthesis of vasopressin analogue chimeric peptides.
3. Complete studies on histone as a potential BBB transport vector.
4. Complete present studies demonstrating the uptake of potential transport vectors such as histone, cationized albumin, or cationized IgG by a number of other organs, other than brain, to elucidate the brain specificity of the various transport vectors.
5. Continue present studies using electron microscopic immunogold to identify the subcellular endothelial organelles involved in peptide trafficking through the BBB.
6. Begin biochemical methods to purify the endothelial transport vesicles that have been identified by electron microscopy.

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